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Biomarkers for the Detection and Measurement of Toxic Effects in
Normal and Malignant Cells from Patients Receiving High-Dose
Melphalan with Autologous Peripheral Blood Progenitor Cell Rescue
in the Treatment of Metastic Breast Cancer

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I.Introduction

A.Nature of the problem

This study provides an extraordinary setting for applying biomarkers of cellular sensitivity to monitor and analyze therapeutic versus toxic effects in cancer chemotherapy. The difference between delivered and biologically effective doses of a drug is the consequence of many complex interacting factors. These include: absorption, cellular uptake, distribution, affinity for intended target tissue and excretion capacities; outcome of metabolic activation processes; ability to repair damage and levels of cofactors that modify drug action. Presently, it is not technically feasible to quantify each of these processes individually and use the sum of such complex information to predict effective doses in a target tissue. A novel approach to the direct detection and measurement of damage to DNA, the ultimate relevant target, however, provides a means to evaluate effective doses of chemical agents with reference to administered dosage and damage to specific target tissues.

B. Background and previous work

Several methods have been used to evaluate levels and persistence of DNA damage, treatment outcomes, and the occurrence of iatrogenic tumors after cancer therapy. These include cytogenetic techniques to characterize chromosomal aberrations and/ or sister chromatid exchanges (Tucker et al., 1990) in peripheral blood lymphocytes (PBL) activated by mitogens and the DNA repair competency methods (Schwartz et al., 1988). They are, however, limited to the use of proliferating cells, require moderate volumes of blood, involve extended processing and are labor intensive. Thus they are generally unsuitable for examining DNA damage and repair among tumor cells or for monitoring levels of damage during the course of therapy.

Among the biochemical methods used to study PBL from individuals exposed to cytotoxic agents are alkaline or neutral gel elution for measuring DNA breaks (Doerjer et al.,1988) and, though they offered significant promise, the gel elution methods utilize pooled cell populations and critical information about intercellular differences in DNA damage and repair is not assessable. Chemical adducts to DNA in cells sampled from patients under treatment have been analyzed by ELISA or by 32-P-post-labeling technique (Perera et al., 1988). The adduct methods fail to yield information on the distribution of damage among cells, are limited to specific sets of adducts, require large cell samples and relatively expensive.

Though a number of point mutation assay systems have been used to detect and monitor genotoxic effects of chemotherapy induced gene damage in breast cancer patients, they are not suitable for real-time monitoring. They tend to be expensive, require large blood samples, lengthy and variable mutation expression times and labor intensive processing (Strauss and Albertini, 1977,1979; Strauss, 1982,1983,1991).

The need for observing the actual concentrations of drugs in cancer patients is undeniable. Achieved blood levels can vary by as much as 6-fold among individuals administered

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identical dosage schedules. In many instances, measures of damage to DNA in tissues, including tumors, may be more informative than blood levels of various genotoxic drugs.

The single cell gel electropheresis assay (SCG), also known as the "comet" assay, has been under development and primary utilization in this laboratory and others in recent years. The complete listing of published work to date in this area is cited in a review article by R.R. Tice, (1995) and by Tice and Strauss, 1995. SCG has tremendous potential for real-time monitoring of DNA damage levels in patients undergoing genotoxic therapies. SCG combines the simplicity of biochemical techniques for detecting DNA single strand breaks and/ or alkali-labile site with the single cell approach typical of cytogenetic assays. The power of the assay lies in its capacities:

     i. to evaluate virtually any tissue containing nucleated cells;
    ii. to study proliferating or nonproliferating cells;
   iii. to study cryopreserved and thawed cells;
   iv. to measure DNA damage (single strand breaks & alkali-labile sites) caused under in vivo or ex vivo exposure conditions;
    v. to measure repair of DNA damage;
   vi. to test extremely small numbers of cells;
  vii. to be performed rapidly (within a few hours of sampling;
 viii. to be performed inexpensively;
   ix. to be performed under crude, even field, conditions;
    x. to measure DNA damage with a high degree of sensitivity
       (X-ray detectable at < .25 Gy or about 200 events/ cell) and
   xi. to evaluate cells for DNA damage individually based on
       antigenic phenotype.

Singh et al. reported the development of SCG in 1988. The microgel alkaline electrophoresis assay was originated to detect and quantify single stranded DNA breaks and alkali labile damage in individual cells. Single cells were embedded in agarose on slides, lysed in situ with detergents, electrophoresed under alkaline conditions (pH = 13), stained with ethidium bromide and examined under fluorescence microscopy. DNA fragments migrated towards the anode with the smallest moving farthest from the nucleus; the image is that of a comet with a brightly fluorescent head and a tail that grows with increasing DNA damage to the cell. The measurements are performed and recorded using computerized video capture and digitization.

The first report by Singh et al. described the ability of SCG to measure DNA damage in PBL exposed with either x-irradiation or hydrogen peroxide (H2O2) each at various doses. Both agents induced damage relative to dose with damage homogeneously distributed among radiation exposed cells but heterogeneously distributed among those H2O2 exposed. Radiation treated cells were examined for repair capacity by raising the temperature of the cell suspension to 37°C and incubating over various periods of time. Within 15 minutes roughly 75% of the DNA repair was complete and by 120 minutes virtually all the repair was done. However, there were a number of cells that were unable to repair at all. Finally, it was postulated that at the lowest dose employed, .25 Gy,

significant DNA damage was measurable and likely to represent about 250 single strand and/ or alkali labile sites per cell.

In the past few years a variety of preliminary applications and refinements of SCG have been reported. These are described in full in our original proposal and in the review article by Tice, 1995. Several findings particularly pertinent to the present research are herein discussed briefly. In a study on mice trapped from a hazardous waste site, Tice et al., 1991 found evidence of minority, heavily damaged subpopulation cells in the bone marrow that, presumably, accumulated over a period of chronic toxic exposure. These unusually sensitive cells were found only after careful dispersion analysis. Previous DNA repair in aging studies by Singh et al., 1990 had revealed, via similar analysis, that a deficient subset persisted and increased with age. Mass assay techniques could not have detected these rare and biologically significant cells. In the present effort we are concerned with the characterization of cellular subsets with regard to their sensitivity to melphalan.

Our laboratories (Tice and Strauss, 1995) have performed several studies with intentions of improving SCG methodology, characterizing its capabilities and further substantiating its ability to evaluate human blood cells. We assessed low-dose ionizing radiation induced damage after ex vivo exposures of human PBL. It was shown that DNA damage among PBL was no more heterogeneous (based on the ratio of range to standard deviation) than that detected among non-irradiated cells. Further, in contrast to chemical exposures, the extent of heterogeneity was independent of the treatment dose. SCG, under performance conditions utilized, was found to be capable of detecting the extent of DNA damage induced by a radiation dose of between 0.013 and 0.049 Gy. This represents a level of sensitivity considerably higher than previously attained with gel electrophoresis techniques.

A further effort in this area considered the magnitude and intercellular distribution of DNA damage induced by low doses of gamma rays in unfractionated blood compared with those observed in isolated pMNC and peripheral polymorphonuclear cells (pPMNC). Irradiation resulted in a linear and dose-dependent increase in DNA damage for all three populations. The dose-dependent increase for DNA migration was not significantly different between separated pMNC and pPMNC, but their responses were significantly elevated over that obtained for leukocytes irradiated in whole blood. Again, as described in the earlier study, the distribution of damage among cells was relatively homogeneous and independent of dose and cell population. These results were consistent with the hypothesis that DNA damage induced in mammalian cells by sparsely ionizing radiation is localized to small regions of the genome and that irradiation of leukocytes in whole blood partially protects against radical-induced DNA damage. We have, however, determined in the course of the present research that when DNA repair is permitted, there is a marked difference in toxic effects on the various leukocyte subpopulations.

We, Tice et al., 1992, applied SCG in its first application in the clinical setting . This preliminary study relied upon the use of cryopreserved peripheral blood mononuclear cells (pMNC) obtained from 11 breast cancer patients before, during and after treatment.. Based on a concurrent analysis of DNA damage in cryopreserved and unpreserved PBL from patients, neither the mean level of DNA damage nor the dispersion of damage among

cells was affected by the process of cryopreservation. The pre-treatment samples of several patients contained PBL with increased levels of DNA damage, presumably reflecting persistent DNA damage caused by induction therapy. High-dose chemotherapy resulted in a significant and variable increase in DNA damage in PBL samples from all patients. Among the post-treatment samples, levels of DNA damage had returned to normal in most, but not all, patients. The presence of damaged cells in the last set of samples may be indicative of an inadequate therapy regimen or of DNA damage resulting from processes unrelated to therapy. As we now are aware, the major effect of cis-DDP on DNA is to produce crosslinks inhibiting the migration of DNA even if fragmented by other agents (perera et al., 1988). cis-DDP levels were not measured. Due to the lack of samples after the final day of treatment, correlation to compare the levels of damage with plasma levels of CTX, lymphocyte toxicity, or treatment outcome were incomplete. The present study is not complicated by interacting toxicants because Melphalan, though it causes heterogeneous damage including some crosslinking of DNA, is used as a single agent.

The British Columbian group, Olive et al., 1992, has used SCG very effectively to investigate some important aspects of antitumor therapeutics. They analyzed etoposide-induced DNA damage at the single cell level showing that sensitivity and resistance were functions of cell cycle condition, i.e., growth fraction cells were damaged and the non-proliferating cells were not. They cited references to the explanation that the non-cycling cells are resistant to killing and DNA damage by etoposide because they lack significant levels of topoisomerase II, the essential target for etoposide-induced DNA damage. The specificity of the etoposide method for measuring growth fraction via SCG was verified directly by allowing cells to incorporate bromodeoxyuridine (BrdU) into DNA, treating with etoposide and labeling the comets with anti-BrdU antibodies. The damaged cells were BrdU positive as expected. The present study employs this BrdU technique to measure DNA damage with reference to the proliferative state of the cells. We hope to gain a greater understanding of the influence of cell growth states on melphalan sensitivity for both normal and tumor cells. We are particularly concerned with the potential effects of extrinsic G-CSF on the toxic and therapeutic effects of chemotherapy.

C. immunobead phenotyping

We developed an immunological phenotyping technique for antigen specific labeling of subpopulation cells that can then be evaluated with SCG as described in Strauss et al.,1994. We suggested that immunobead method may be applicable to biomarker approaches capable of assessing individual cells as an adjunct to determine effects of exposures on specific subset cells within heterogeneous populations.

Suffice it to say here, the immunobead typing method works because cells and associated beads are immobilized in the gel prior to detergent and alkali treatments; their spatial relationships are maintained despite digestion of protein and RNA. A further consideration determining whether this approach can be validly employed is whether the microspheres affect the migration of DNA particles during electrophoresis. Studies were performed in our radiation effects series, Vijayalaxmi et al., 1992, 1993, to test and validate the methodology. Ionizing radiation effects were examined in terms of DNA damage and

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repair in specific subpopulations of peripheral blood leukocytes. Essentially identical distributions of DNA migration lengths were observed for beaded and non-beaded cells both for unirradiated and irradiated T cells. In addition, no correlation was found between migration length and number of beads per cell indicating that the presence of beads did not influence DNA migration. The method and its preliminary exploitation was described more fully in the original proposal. Some important new advances have been made in this area in the course of the present research and are described herein.

Studies were conducted in this laboratory to compare DNA damage and repair in (naturally occurring) heterogeneous mixtures of normal BM-MNC and breast carcinoma micrometastases following ex vivo (chemical purging) exposure with 40 ug/ml 4-hydroperoxycyclophosphamide (4-HC). Though rare, tumor cells are detectable in BM from many breast cancer patients with metastatic disease. Artificial mixture experiments estimate resolution at about $5.0 \times 10-6$ tumor cells in normal BM. We have successfully applied SCG to assess normal and tumor cells, side-by-side on the same slides. BMs were labeled with a monoclonal (317G5+) against breast cancer specific antigens, marked with the 4.5 micron microspheres, blocked with goat anti-mouse monoclonal antibody and then, to rule out nonspecific labeling, they were counter-labeled with monoclonal CD-45 against early common leukocyte antigens, and then marked with sheep anti-mouse antibody coated 2.8 micron beads. The 2 sets of beads were easily and unambiguously distinguishable by size. In addition, cells from the breast cancer line, CAMA-1 were similarly marked, either exposed with 4-HC or unexposed, allowed 30 minutes repair time or none and tested by SCG.

It was found that each cell population was heavily damaged by 4-HC exposure and that repair occurred to various degrees according to qualities inherent to each. Briefly, after a repair period of 30 minutes, cells repaired to within 69% of the untreated value for normal BM, 20% for metastatic breast cancer cells and 6% for CAMA-1. These experiments were more completely described in the original proposal and in Strauss et al., manuscript in preparation. In this series we have also examined 4-HC dose-effect relationships using a highly sensitive fluorochrome-mediated viability assay (FMVA) (Strauss, 1991) differentially to assess normal BM and immunobead marked tumor cells for both viability and DNA damage. There are, not unexpectedly, strong correlations between viable states and degrees of DNA damage and reparability (Strauss et al., manuscript in preparation).

Our laboratory conducted modest studies to compare specimens from FNA and excision biopsy of the same tumor in several patients. The specimens were characterized according to methodology of preparation in terms of morphology and phenotype, cell number and viability. In vivo condition for each specimen was assessed by SCG and FMVA. Ex vivo exposures with 4-HC were then conducted to measure dose range effects on viability and DNA damage and repair. Data for each specimen type were similar in many respects, however, the mass biopsy procedure was more difficult for the patient (and staff) and sample preparation required more bench time. FNA produced fewer tumor cells, but yields were more than sufficient for the studies planned. FNA is, therefore, our tumor sampling method of choice for serial sampling from patients before, during and after treatment of

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patients. We believe FNA will provide ideal specimens for the studies of drug resistance to be proposed.

It is known that tumor glutathione (GSH) concentration is an important factor in resistance to alkylating agents in chemotherapy. Hercbergs et al., 1992, demonstrated that erythrocyte (RBC) GSH concentrations are related to clinical response in patients receiving their first courses of therapy. They inferred from this that erythrocyte GSH concentration might correlate with that of tumor cells. They suggested that RBC GSH evaluation might allow prediction of the probable response to chemotherapy and identification of candidates for glutathione depletion therapy. As, they pointed out, many studies have reported the use of buthionine sulfoximine (BSO) to deplete cellular GSH in vitro experiments. BSO is a specific inhibitor of gamma-glutamyl cysteine synthetase, which is a key enzyme in the synthesis of GSH (Batist et al.,1986). Koberle and Speit, 1990, e.g., showed that reduced GSH results in potentiation of 4-HC cytotoxicity. In tumor bearing mice, melphalan treatment was markedly more effective after GSH depletion. We recently concluded pilot studies to define conditions for measuring GSH depletion effects on patient specimens and report the results in this presentation.

Finally, the proposal shows results from a pilot experiment that measured plasma levels and DNA damage in pMNC by SCG in a patient receiving high-dose melphalan alone on the first day of a multiple drug (ICAM) treatment for metastatic breast cancer. This aspect of the work has been advanced considerably and representative results are presented herein.

II.Body

A.Objectives

The purpose of the present work has not been altered appreciably from that proposed and can be summarized as follows:

1. To establish a battery of assessment methods with which to monitor therapeutic and toxic effects in cancer patients undergoing treatment. The ultimate goal is to develop a system for ex vivo predetermination of optimal agents and doses for each patient as an individual.
2. To establish appropriate procedural controls assuring the quality of assessments to be performed, in this and future efforts, using the battery of assessments proposed.

3. Further to characterize, improve and validate SCG for monitoring cytotoxicity in cancer therapy. For example, this study presents an opportunity to observe latency and patency periods for SCG in vivo.

4. To compare individuals in terms of intrinsic sensitivities of their normal and tumor cells.

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B. Methods refresh

1. Status of the Clinical Trial

The project drew samples from 3 different clinical trials run through the Duke University Bone Marrow Transplant Program. The first trial was the immuno-pharmacologic purge trial in which 4HC was used to purge the bone marrow of contaminated breast cancer cells. This provided a unique opportunity to study the differential effects of 4HC on normal and malignant cells. The second trials was a Phase I/II ICaM (High dose Ifosamide, Carboplatin and Melphalan) for patients with metastatic breast carcinoma. The ICaM patients received high dose Melphalan on day -7 prior to the Ifosamide and Carboplatin, so provided samples both pre and post Melphalan in the absence of other alkylating agents. The third clinical trial was the Melphalan trial which patients received 2 or 3 cycles of high dose Melphalan supported by peripheral blood progenitor cells. The Melphalan trial is for patients who are not eligible for our clinical trials, i.e. have been treated in the metastatic setting or were refractory to induction chemotherapy.

Thee three clinical trials are funded by other means, and NCI Program Project Grant. All data generated by the clinical trial are shared freely among various investigators. There are many no cost benefits for the present project and because of its adjunct role, there were certain restriction applied to manner in which the data are to be released.

As also discussed in the results section, data from the pharmacokinetic assays on plasma melphalan levels are not currently reportable, though we have them on our spreadsheets and have started our analyses of them. Benefits, however, far outweigh this temporary limitation. These include the valuable services of the BMTP's dedicated data management section providing expert database support and analyses by qualified biomedical statisticians. Specimens are to be obtained from each patient accrued by the clinical trial and subjected to testing according to the schemes and techniques presented in this section. Experimental approaches for assessing specimens after in viva or in vitro exposures are discussed and depicted below and in figures 1 and 2. The discussion describes several adjustments made to the plan and methodologies originally proposed.

2. Testing Schemes

a) In vivo Exposures.

Samples of the listed specimen cells are studied at times before, during and after each treatment cycle as shown here in outline form. WBC from peripheral blood, normal BM and metastatic breast tumor cells from bone marrow, and metastatic breast tumor and WBC from solid tumors are each being evaluated for: a) DNA breaks by SCG; b) proliferative index by BrdU incorporation and X-BrdU labeling; c) viability by FbIVA and d) erythrocytes are to be tested for GSH levels.

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Cell Type (immunotype) and sampling times:

   A. Peripheral Blood -- (pMNC (CD-5) \ pPMNC (CD-15)
      1. sample: day 0 (day of melphalan infusion), 0 min
         (pre infusion), 30 min (mid-infusion), 60 min
         (end-infusion), 75 min, 90 min, 120 min, 180 min,
         300 min, 420 min; & day 11 of each cycle
            (times 3 cycles)

   B. Bone Marrow -- (CD-45 \ Ca Moabs)
      1. sample: day 0, pre Rx; & day 11 of each cycle
            (times 3 cycles)

   C. Tumor (FNA)   -- (CD-45 \ Ca Moabs)
      1. sample: day 0, pre Rx; day +1
            (times 3 cycles)

   D. Erythrocyte
      1. sample: day 0, pre Rx; & day 11 of each cycle
            (times 3 cycles)

Figure 1.

IN VIVO EXPOSURES	ASSESSMENT			SCG / Repair	Proliferation Index		GSH	Viability		Smears Histo-stain & Ca Moabs/CD-45
	SCG									
				vitro	vivo	vitro		vivo	vitro	vivo
EXPOSURE CONDITION	vivo	vitro	DNA	vitro	vivo	vitro				
SPECIMEN TYPE										
Blood, CD-15, CD-5										
p-RBC										
BM, CD-45										
BM, Ca Moabs										
p-Prog, CD-34										
p-Prog, Ca Moabs										
FNA, CD-5										
FNA, Ca Moabs										

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b) Ex Vivo Exposures:

Effects of ex vivo exposures are being assessed as for in vivo exposures with the addition of the repair component measured via SCG. Cells being studied are cryopreserved aliquots of PBL and BM from the same normal individual; cryopreserved aliquots of CAMA-1, the breast cancer line; freshly sampled tumor and WBC by FNA; and peripheral progenitor cells. The original plan included testing of rare, contaminating metastatic tumor cells from the peripheral blood. Our harvest protocol, however, now calls for selection of CD-34 rather than tumor. It is more reasonable for us isolate the more common BM metastatic cells. Each cell type is being exposed and tested intact. We had planned to test another set, as naked DNA, however that now appears to be unnecessary based on results collected to date. The primary goal of testing naked DNA was to render the specimen DNA sensitive enough to measure melphalan activity from the plasma. This is because the pharmacokinetic readings indicated very low concentrations at critical points during and after administration of the drug. Surprisingly, even inactive, normal control PBL (CD-5) were perceptibly damaged by patient plasma containing as little as 1.0 ug/ml melphalan.

The exposure agents, being used separately against each test cell, are G-CSF, melphalan, plasma and BSO. The G-CSF and melphalan are used at concentrations approximating those achieved physiologically. The samples of plasma will have been frozen at the times listed below and are thawed at the time of testing.

Cell Type --immunotype, sampling times
    A. PBL, standard normal   (frozen aliquot)
    B. BM, standard normal   (frozen aliquot)
    C. CAMA-1, breast ca. line   (frozen aliquot)
    D. FNA   -- 3 X-ca Moabs \ CD45+   (fresh biopsy)
        1. sample: day 0, pre Rx; day +1
            (times 3 cycles)
    E. p-Progenitor -- 3 X-ca Moabs \ CD34+   (fresh harvest)
        1. sample: day (-4,-5 or -6), pre Rx

  Exposure condition of cells
        1. Intact cells
        2. Naked DNA (SCG only)

  Exposure agent
        a. G-CSF   (initially titrate to find relevant dose)
        b. melphalan   "
        c. plasma*   "
          sample: day 0 (day of melphalan infusion), 0 min
              (pre-infusion), 30 min (mid-infusion), 60
              min (end-infusion), 75 min, 90 min, 120
              min, 180 min, 300 min, 420 min; & day 11
              of each cycle (times 3 cycles)
        d. BSO, then plasma or melphalan   * from frozen specimens

Figure 2.

IN VITRO EXPOSURES		ASSESSMENT			MODALITY					
		SCG			SCG / Repair	Proliferation Index		GSH	Viability	
EXPOSURE AGENT	SPECIMEN TYPE	vivo	vitro	DNA	vitro	vivo	vitro		vivo	vitro
plasma	MCF-7 cell line									
melphalan	MCF-7 cell line									
plasma	PBL, standard nl									
melphalan	PBL, standard nl									
plasma	BM, standard nl									
melphalan	BM, standard nl									
plasma	FNA, Ca Moabs									
melphalan	FNA, CD-5									

A number of alterations in the methodology as originally proposed have been instituted as a result of observations from work in progress. These are now described in context of the methods and materials as proposed.

3. Exposure agents

Melphalan: Solutions are prepared in 75% ethyl alcohol containing an equimolor concentration of hydrochloric acid. Further dilutions are made in aqueous medium immediate prior to use to minimize hydrolysis. Doses for in vitro exposures were titrated in preliminary studies and are based on an observed average peak plasma concentration of approximately 6.5 ug/ml. The observed peak concentration from the patients appears now to be slightly lower than expected, about 5.0 ug/ml. The standard curve utilized easily encompasses the observed range. The source of melphalan used is Sigma Chemicals, not the product from the treatment vial originally tested and the drug prepared for the exposure studies was aliquoted to 75 separate vials and immediately cryopreserved at -80°C. The melphalan challenge doses used in the ex vivo exposure studies are centered around 4 and 8 ug/ml.

G-CSF (Sandoz): G-CSF was titrated (at doses escalating from 5.0 ng/ml) against activity as measured by proliferation index in p-progenitors. Proliferation index of each cell type as affected by G-CSF stimulation was measured by BrdU incorporation. Indices of DNA

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synthesis were compared contemporaneously with degree of damage determined by the assessment modalities for effects of in vitro exposures.

Plasma: 5.0 ml venous blood are being obtained from patients in .2% Na EDTA-coated vacutainers at times indicated above. The tubes are immediately refrigerated with subsequent separation within 12 hours and quantitative analysis within 48 hours. A 1-2 ml plasma sample from each specimen is frozen for later use in vitro exposures of intact cells or naked DNA. The cellular fraction of each separation (by simple centrifugation at 100 xG) is studied, as described in the in vivo exposures section above, for GSH content (erythrocytes) and for the remaining 4 assessments (pMNC \ pPMNC). It should be pointed out that a change has been implemented in the identification of the pMNC fraction. Rather than simply counting cells not CD-15, we double label CD-15 and CD-5. Therefore the pMNC fraction measured is a specific subset (T cells) and results are not open to variability between pMNC subsets including B cells, null cells and monocytes. Each of these could be differentially sensitive to melphalan, or the response modifiers, and a mere fluctuation in relative ratio could yield misleading data.

BSO: Butathionine sulfoximine (Sigma) Doses for in vitro exposures are to be titrated in preliminary studies based on the use of 50 uM / 1 hour exposure time at 37˚C. This incubation is followed with a washing step and then treatment with CM, CM + melphalan or CM + plasma; then further incubation at 37.C, 30 minutes, adjustment of cell density and plating for electrophoresis. The first study of BSO mediated modification of sensitivity to melphalan has been completed. It was based upon a large and related study of MDR cell lines and the modification of sensitivity to arsenic, 4-HC and melphalan (Everson et al., manuscript submitted to be cleared for publication). Breast cancer cell lines, MCF-7 and ADR-10, wild type and drug resistant, respectively, were acquired and cultured as described below. It was determined that preincubation of cells with 25 ug/ml BSO for at least 48h is sufficient to render the resistant strain considerably more sensitive to the effects of melphalan. BSO incubation was followed with the detachment step. Further testing is being conducted to measure modifications in resting PBL, BM and proliferating p-Progenitors.

Sampling & handling: Blood specimens are held at 4.C until separated into plasma, pMNC/ pPMNC, and RBC fractions. BM, FNA and p-p-prog specimens are transferred at 1:1 (v:v) to tubes of complete medium containing 10 uM BrdU and held at 37.C for 60 minutes, to allow incorporation into DNA, and then further processed. Thus far, the latter specimens have been tested as described below.

4.Revised Phenotyping techniques

Antibodies: Anti-breast cancer monoclonal antibodies (Moabs): 317G5+,260F9+,520C9+ (Cetus)(Ca Moabs). These have been in use in the BMTP at Duke and their use in immunomagnetic purging (IMP) and identification have been reported over the past 5 or 6 years. The following Moabs to human leukocyte markers (CD antigens) (Sigma Immunochemicals) are applied: CD-15 (p-PMNC); CD45+ (BM- leukocytes) and CD34+ (p-progenitors). Goat antimouse IgG for blocking in double labeling (Sigma).

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Additionally, as mentioned previously, CD-5 (T cells) has also been added to our phenotyping inventory.

Significant technical advances have been made in the use of phenotyping techniques (originally developed in our laboratory). The improved methodologies have resulted in improvements that conserve time, money and yield greater consistency in the data. The revised technique consolidates the parts of the original by, in essence, allowing the 1st and 2nd antibodies to recognize each other in advance of presenting antigen. The steps are simplified and, paradoxically, the produce is enhanced.

Microsphere labeling: Immunomagnetic beads, 2.8 and 4.5 micron Dynabeads (Dynal) covalently coated with sheep anti-mouse IgG are twice washed and resuspended in PBS/FBS. 4.5 micron beads or 2.8 micron beads are coated with the 2nd antibody added slightly in excess. About 50.0 ul Moab (at ~200 ug/ml stock) are added to 1.0 ml bead suspension and incubated on a rocker for 2 hours at room temperature. The beads are washed well in cold PBS containing 10% complement inactivated fetal bovine serum (PBS/FBS) and stored in clearly marked vials. At the time of testing the beads are added at about 10 per cell and incubated 20 minutes at 4°C. Cells are then resuspended and agar embedded, or smeared and stained, or exposed with exposure agents and embedded, etc. and further processed. Beads of both sizes bearing Moabs of specificities that will distinguish cells of subsets may be added simultaneously. This method has improved our results immensely and we intend to publish it as a brief methodological article showing data from some of the experiments described below.

5.Testing methods

DNA repair: In the absence of inhibitors, this is accomplished after exposure with a DNA damaging agent simply by allowing suitable conditions and time in which for repair to occur. After exposure and washing cells are allowed 0, 30 and 60 minutes at 37.C and then they are processed further by the SCG procedure. The 120 minute repair time point has been deleted as an unnecessary variable in the course of the present studies.

Naked DNA exposures: SCG minus the repair component can be used to assess naked DNA. For this purpose, untreated cells can be embedded in agarose, then exposed with the test agents (on separate slides), then the second layer of low melting point agar applied and the SCG completed as usual. As discussed previously, it now seems unnecessary to test in this manner because the primary projected need for this technique appears to have been obviated. The plasma melphalan from patients is biologically recognizable at relevant levels in the standard assay. A drawback to the approach for present purposes was the inherent difficulty in delivering a particular volume and contents evenly over a slide and through gel.

Slide culture: If the proliferation index in harvested CAMA-1 cells (the same could apply for MCF-7 cells) is insufficient an hour or so later, it would be necessary to allow cells to adhere to sterile frosted slides in petri plates over a period of time to permit log phase growth. These would then be exposed on the slides and assessed "in situ". This technique

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is now known to be unnecessary on the basis of preliminary proliferation assessments by the Brdu method.

Proliferation index: Cells in S-phase are identified by incubating specimens with 10 uM BrdU, 60 minutes at 37.C and, following electrophoresis, labeling 20 minutes, at 4.C with FITC-anti-BrdU (Becton-Dickenson) at a 1:50 dilution with PBS. Cells that incorporate BrdU are clearly marked green (at 488 nm excitation) on a background of red (at 546 nm) (ethidium bromide) and are scored using a - / +/- /+ system. This method performs fairly well although it is necessary to put the bias against the positive and negative cell by scoring +/- unless there is no doubt whatsoever that a cell is -/ or +/ . On SCG, cells that are damaged and proliferative appear to be yellow as they fluoresce both red and green. Again one can afford to score with utmost certainty because finding 100 cells to count amongst no less than 10⁻4 is not problematic.

Glutathione levels: The method of Beutler, 1984, is to be applied to measuring erythrocyte GSH. .2 ml whole blood is added to 2 ml of distilled water, .2 ml of the lysate is added to 10 ml of ferricyanide-cyanide reagent for hemoglobin estimation, and 3 ml of precipitating solution is added to the remaining 2 ml hemolysate. After 5 minutes, the mixture is filtered, 2 ml filtrate are added to 8 ml of .3 M Na_2HPO_4 solution in a cuvette. It is read at 412 nm against a blank (2 ml of 2:5 water diluted precipitating solution to 8 ml of phosphate solution. A second OD is done after 1 ml of DTNB reagent has been added to the blank cuvette and the cuvette containing filtrate. A hematocrit reading can be used instead of, or in addition to, the hemoglobin estimation. The procedure are performed at room temperature and the red cell lysates are read within minutes to avoid oxidation. We have performed pilot studies to test the technique, but are saving the frozen specimens to test in a single run to eliminate between tests error.

Melphalan levels: Plasma samples will be collected at times indicated under Exposure Agents in the Methods section above. Samples are immediately refrigerated with subsequent separation within 12 hours and quantitative analysis within 48 hours. Plasma melphalan are determined by a computer-assisted isocratic reverse-phase HPLC assay with UV detection (Waters, Inc.) as described in Peters et al., 1989. The standard curve for this assay is expressed as a linear function over the concentration range of 0.5 to 5.0 ug/ml. The lower limit of quantitation is 0.5 ug/ml with an intra-assay CV of less than 10%.

6. Cultured cells

MCF-7 and ADR-10 Cell Lines: MCF-7 cells originated from a previously untreated carcinoma of the breast. The multidrug resistant cell line MCF-7, ADR-10 was a gift of Dr. Ken Cowan, NCI, Bethesda, MD. MCF-7 cells have been widely used in clonogenic assays and, here, were tested in artificial mixture experiments to evaluate limits of detection by phenotype. In exposure studies with melphalan, patient plasma, BSO and G-CSF they are used to assess sensitivity to these agents. It is of particular interest to determine to what extent the cell line is a suitable surrogate for the wild type tumor in its various manifestations.

MCF-7 Culture Conditions: MCF-7 breast cancer cell lines were cultured in Iscove's modified Dulbecco's medium (GIBCO-BRL) supplemented with 10% heat inactivated fetal calf serum (Hyclone). The MDR phenotype of this cell line was selected by stepwise exposure to doxorubicin (Batist et al. 1986) and was maintained by one week exposure to 10^{-5}M doxorubicin every two months. All experiments involving ADR-10 cells were performed two weeks after the last selection for each drug resistant cell line.

Exposure Conditions: MCF-7 derived lines were grown to from 75-90% confluence and detached with using warm trypsin-free detaching medium (GIBCO), a "rubber policeman" and gentle agitation. Production of a single cell suspension was confirmed microscopically. The detached cells were then washed with PBS, adjusted to a density of 10^6/ ml and exposure agents added as indicated below.

7. Statistical analysis:

The effect of dose or condition on the average length of DNA migration as well as on the intercellular distribution of migration patterns are be analyzed using appropriate on-tailed or two-tailed parametric or non parametric statistics, depending on the statistical nature of the variates. For all statistical analyses, significance are determined at an alpha level of 0.05. The intercellular distribution of damage within a individual sample are evaluated using the dispersion coefficient H, where H is the ratio of the sample variance to the sample mean. H can also be used to determine whether the distribution of damage among cells within a sample fits a Poisson model, the dispersion test. In this approach, the statistic $T = (r-1)$ H, where r is the number of cells analyzed, is calculated. The statistic T is referred to a table of critical values for the Chi-square distribution with r-1 degrees of freedom. Analysis of variance and covariance and multiple linear regression analyses are used to determine the correlation between chemotherapy-induced DNA damage in the various cells and various pharmacokinetic and toxicity-related parameters.

Being concerned with a lack of consensus among researchers handling SCG data, we have sought measures to correct the deficiency. We are consulting with several agencies in this regard and have undertaken a workshop approach to dealing with the problem. This is, of course, a situation common to all new areas of exploration and at the moment, the prospects for significant progress are considerable.

Methods applied in our studies and not mentioned in the previous section have not been altered and are in use as described in the original proposal.

III. Results and discussion

A. General Considerations

In the first term of the project one was particularly concerned with attending to tasks of quality assurance. In projects designed to develop and employ new methods this is especially important and also productive. This process is often referred to as "characterizing the approach." Modifications to some methods, usually on the order of

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"fine tuning," were tried and implemented or abandoned. In one case a new, highly regarded method (immunotyping with microsphere markers) developed in our laboratories was actually improved significantly.

In 2 cases methods proposed as backup options may no longer be needed. "Naked DNA testing in SCG" is the first of these. This special, more difficult method may be unnecessary owing to unexpected success in our ability to test patient plasma containing processed melphalan via ex vivo exposures of normal test cells followed by standard SCG assay. The second backup method that may not be needed is the slide method for growing adherent cells to be tested "in situ" on the slide. This will not be needed because the MCF-7 cell lines can be treated in suspension before SCG or FMVA testing.
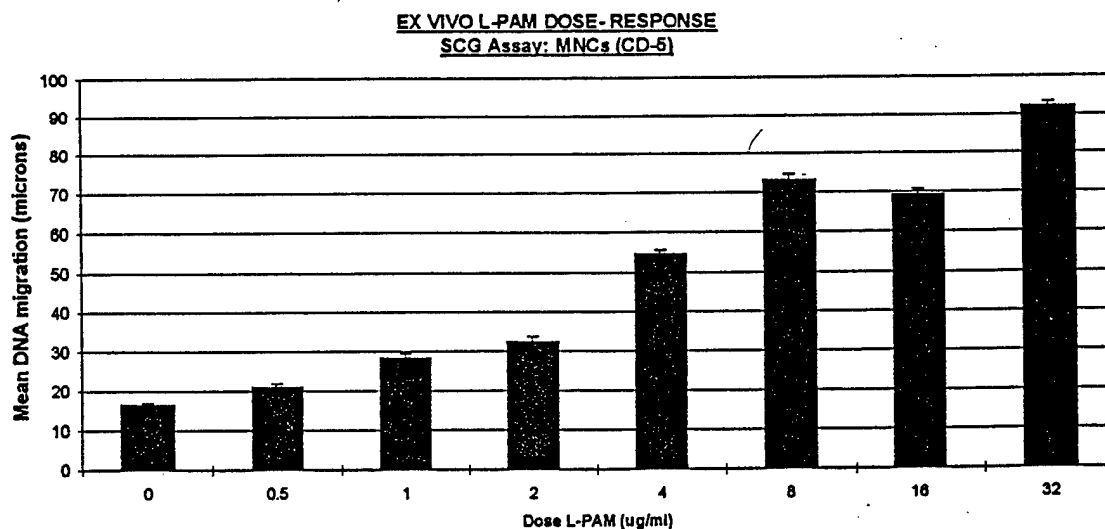
The overall attack strategy has been to standardize techniques, assess and, to the extent possible, eliminate 'between tests' error.

Figures 3 and 4 depict results of repetitive SCG testing. The goals were 1.) to determine, by titration, dose levels appropriate to our purposes and 2.) to monitor for consistency and overall technical quality of the assay.

B. Specific Experiments

1. Melphalan dose- response testing of normal CD-5 cells

Figure 3.

EX VIVO L-PAM DOSE- RESPONSE
SCG Assay: MNCs (CD-5)
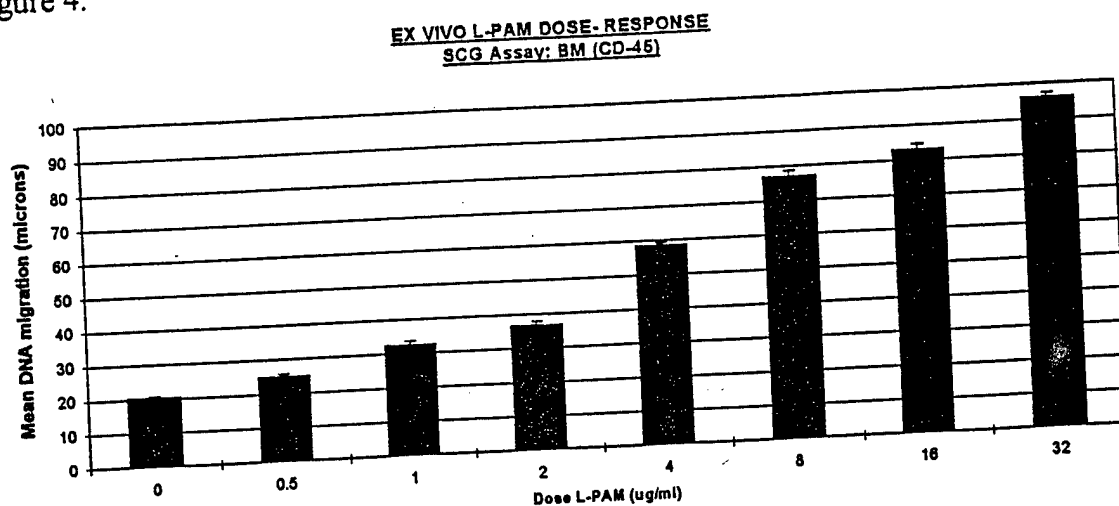
The normal control PBL and BM from cryopreserved aliquots, described above, were phenotyped for CD-5 and CD-45, respectively and, in separate vials, exposed with the concentrations of melphalan shown on the X -axis. Cells were exposed for 30 minutes at 37°C, washed and processed for SCG.

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2. Ex vivo melphalan dose- response – SCG assay of normal CD-45 cells

Figure 4.

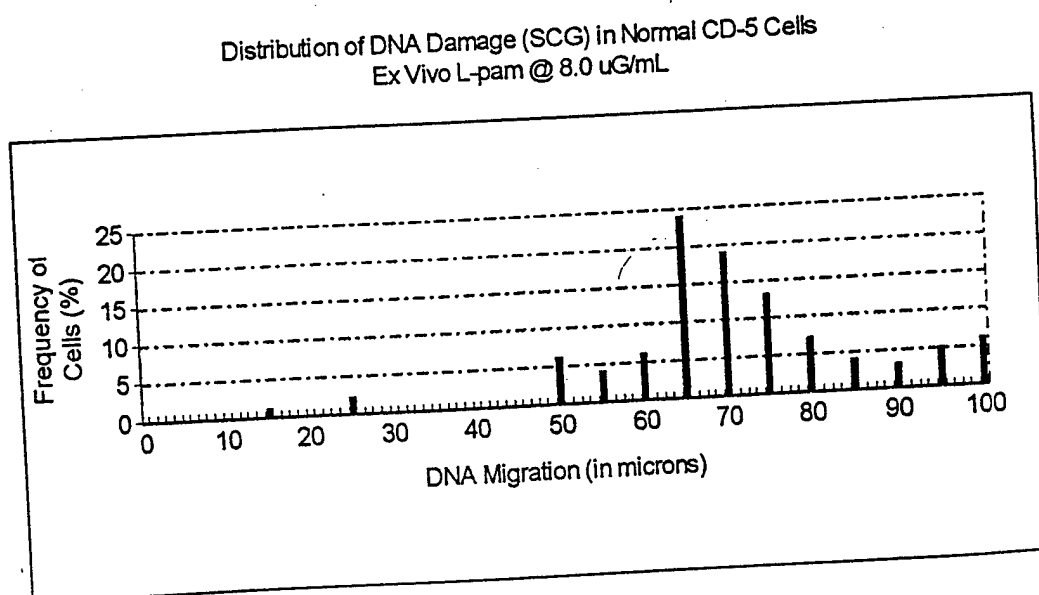**EX VIVO L-PAM DOSE- RESPONSE**
SCG Assay: BM (CD-45)

Data are plotted as usual for SCG showing mean DNA migration in microns on the Y-axis against doses of melphalan in ug/ml on the X-axis. The error bars indicate SEM.

The results show a high degree of reproducibility between the experiments performed over the course of several weeks. As we have previously observed, BM is slightly more sensitive to damage from melphalan than is the T cell subset from peripheral blood. The preparation of melphalan used here was subject to a higher degree of standardization than in previous studies and probably also accounts for more reliable results. 4 to 8 ug/ml melphalan have being used in exposure studies for calibrations and a dose of 2 ug/ml is used as the positive control where patient plasma is the exposure agent.

3. Frequency distribution of DNA damage for normal CD-5 cells exposed to 8.0 ug/ ml melphalan ex vivo

Figure 5.

Distribution of DNA Damage (SCG) in Normal CD-5 Cells
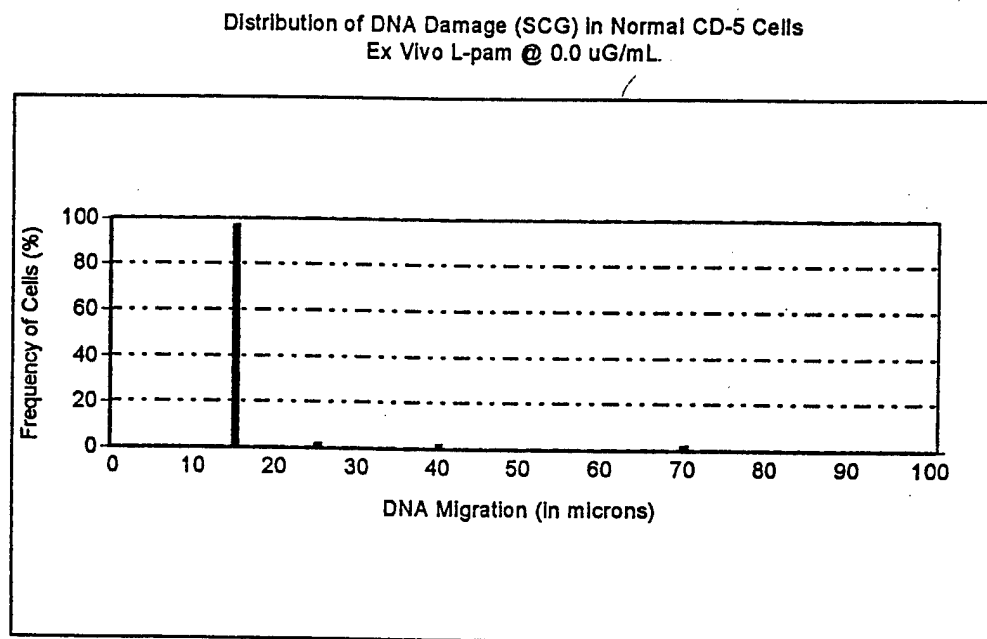Ex Vivo L-pam @ 8.0 uG/mL

Data from 1 slide (100 cells scored) that contributed to the 8.0 ug/ml dose effects shown in figure 3 are presented in figure 5. The intercellular distribution of DNA damage among T cells is shown. Frequency of cells by percentage is plotted on the Y-axis and DNA migration in microns on the X-axis. The mean DNA migration value for this data point was 70.6 +/- 1.531, H= 3.29 versus the unexposed value of 15.9 +/- 0.609, H=2.31. It is not excessively heterogeneous or multimodal, but it is interesting to note there is 1 cell that shows a normal value of 15 microns (no migration from the nucleus) and 2 others that have short tails of 10 microns. These cells are apparently different from all the rest in that they show little or no damage. The significance of the minority subset cells requires further study. One should note that they were not allowed an opportunity for repair as we now understand it to work in the ex vivo setting.

The untreated cells from the same experiment were distributed as shown in figure 6. This is typical of normal, unexposed CD-5 cells. Ninety seven of 100 cells show no evidence of damage with nuclei measuring 15 microns. There is a minority subset containing cells with damage: one each having migration lengths of 25, 40 and 70 microns. These too, are of interest and will be studied further. A sufficiently large database will elucidate the significance of these outlyers. It is interesting to note that this set was put in a condition permissive of repair when they were incubated at 37°C along with the treated group. If the observed damage occurred in the bloodstream or in handling one assumes that repair would have been attempted and that this is residual, persistent damage.

4. Frequency distribution of DNA damage for normal CD-5 cells exposed to 0.0 ug/ ml melphalan ex vivo
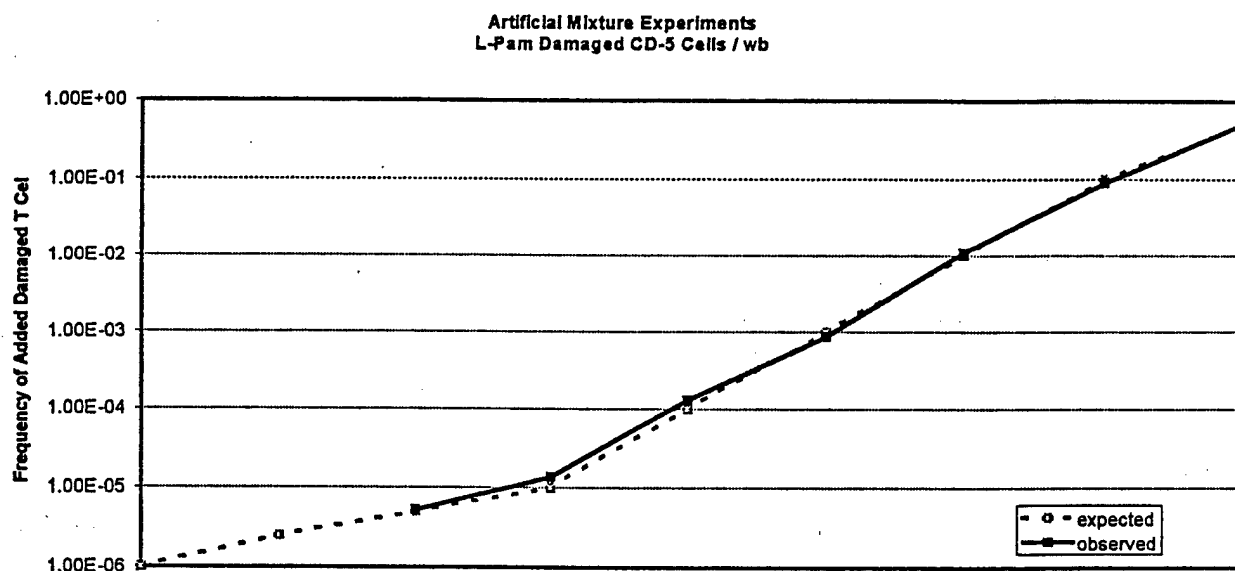
Figure 6.

Distribution of DNA Damage (SCG) in Normal CD-5 Cells
Ex Vivo L-pam @ 0.0 uG/mL

Experiments were conducted further to test technical competency, detection limits and reproducibility of results. Artificial mixtures were made using CD-5 cells heavily damaged at a level of 8.0 ug/ml melphalan and added at log dilutions to whole blood. The results are shown above in figure 7. Expected frequencies are plotted against observed frequencies from 10^{-6} to unity.

5. Artificial mixture experiments—melphalan damaged CD-5 cells in whole blood

Figure 7.

Artificial Mixture Experiments
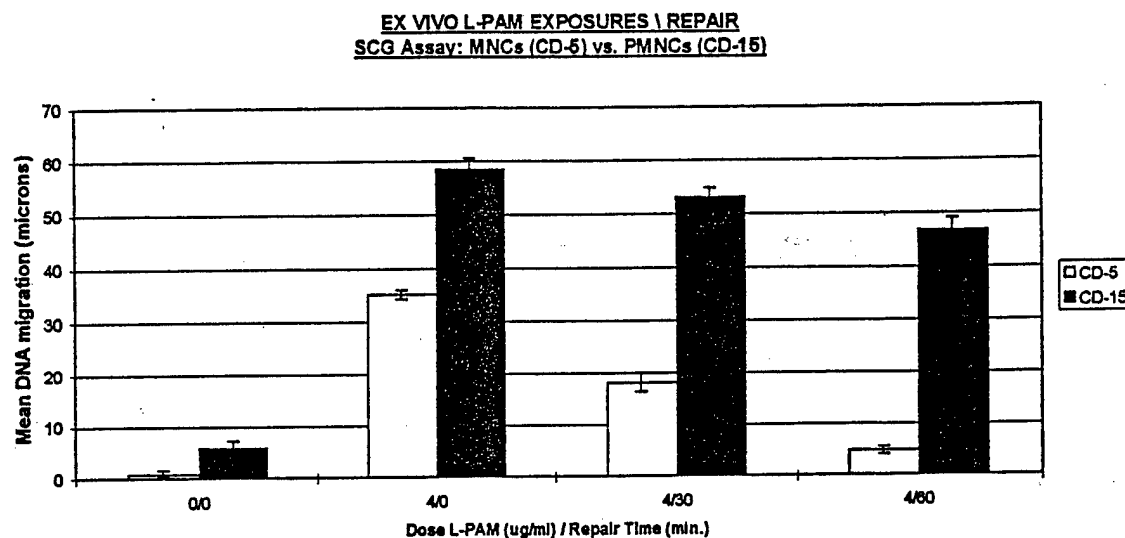**L-Pam Damaged CD-5 Cells / wb**

This experiment was performed several times with virtually identical results (one representative test is shown). Recovery in every case was successful to a level of slightly less than 5.0×10^{-5}. In these studies enumeration methods developed for use in a specific locus mutation assay (Strauss and Albertini, 1977) were used to estimate the denominator representing the vast majority of undamaged cells. In these cases the number of cells present in the gels were estimated from cell counts per volume and volume added. Whole slides were then scanned to pick out the rare damaged cells. It should be pointed out that the naturally occurring damaged cells (as found in the control set shown in figure 6) were accounted for and factored out. This study and another, described below, provided en-

hanced confidence in our technical abilities and allowed for adjustments that have improved overall performance.

6. Ex vivo melphalan exposures and repair - - SCG assay of CD-5 versus CD-15 cells

Figure 8.

EX VIVO L-PAM EXPOSURES \ REPAIR
SCG Assay: MNCs (CD-5) vs. PMNCs (CD-15)

A study to compare responses to melphalan at a level of 4.0 ug/ml among PBL was performed. Beads carrying Moabs against CD-5 and CD-15 were used to mark PBL in whole blood. The blood specimens were next either incubated with melphalan or not. They then were either not allowed to repair or allowed to repair for 30 or 60 minutes and subjected to SCG. The various combinations employed are presented on the X-axis of figure 8 above. The results in figure 8 are plotted as usual for SCG data and show tail length only. In this technique of data analysis, the diameter of the undamaged nucleus for the particular cell type is subtracted out. This approach to scoring allows for comparisons of cells with different sized nuclei.

These studies also tested the practicality of the new phenotyping technique. Prior to spreading for SCG, cells were removed from the vials, serum added, smears made on plain microscope slides and histochemically stained. A total of 16 slides were scored for immunotype and morphology. The incidence of nonspecific labeling was determined to be less than 2% (data not shown).
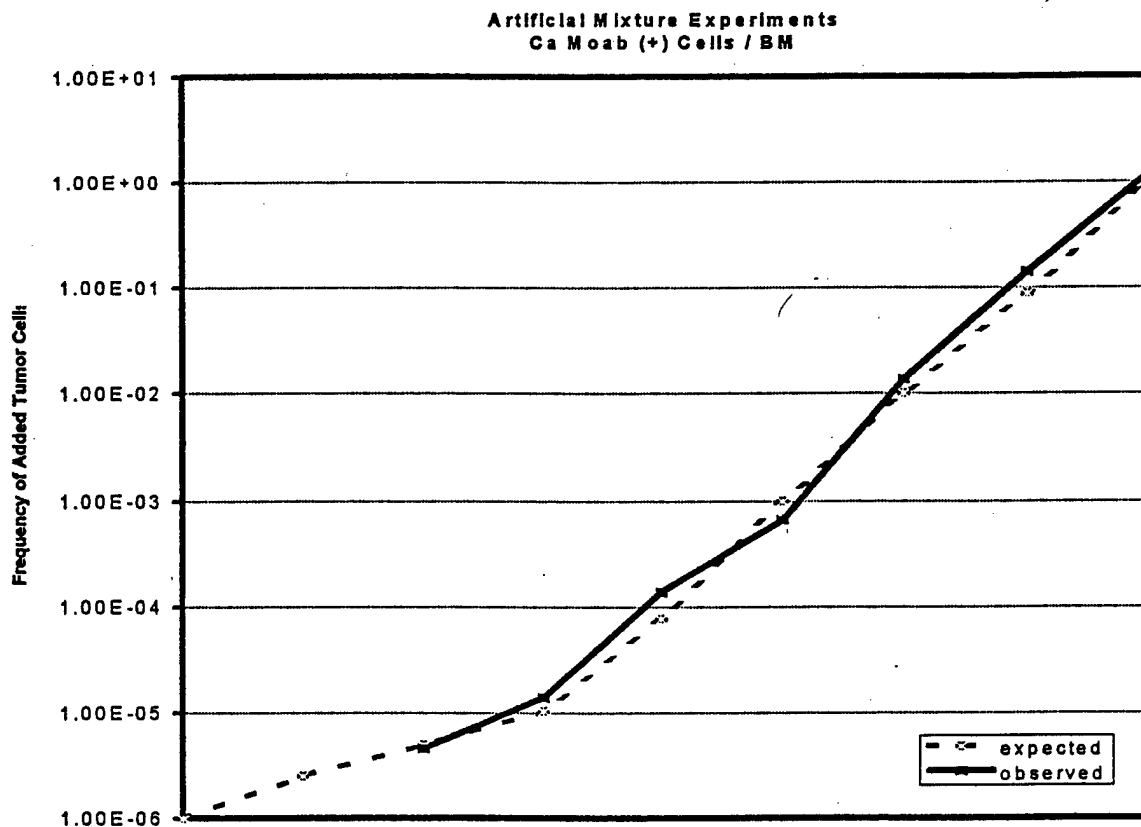
The results clearly indicate that p-PMNC are more sensitive to initial DNA damage from melphalan than are T cells. The latter are considerably more repair sufficient than the former. The extent of repair was 49% from 0'to 30' and 39% from 30' to 60% or a total of 87% for the CD-5 set. The total repair for the CD-15 set was 22%, 10% from 0' to 30' and 12% from 30' to 60'. We have suggested in previous reports (Tice and Strauss, in press, Stem Cells) that granulocytes have no need for repair considering their half-life of ~5 h and the fact that they carry myeloperoxidases released in the event of cellular damage and causing further DNA breakage in self and surrounding cells. This study demonstrates the need to score subset cells differentially in their natural heterogeneous mixtures. As

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mentioned previously, mere changes in ratios of differentially sensitive subset cells will certainly squew results for uncontrolled mixtures. The ability to score subsets side by side in situ is an important advance in our research.

A series of artificial mixture experiments were performed in a manner similar to those illustrated by figure 7. In this case the added minority cells were bone marrow metastatic breast cancer cells purged from bone marrow harvests using the immunomagnetic purging technique described in the proposal. The cells were marked by beads which recognized the 3 antibodies of our Ca Moab panel which had attached to the tumor cells in the BM. They again were added at various log dilutions down to 10^{-6}, this time to a majority population of normal control BM. The slides were scored in the same manner as described for the previous but they were not electrophoresed as no DNA damaging challenge agents were applied. The ability to score beaded cells was tested and the results again indicated recoveries at slightly better than 5.0×10^{-5}.
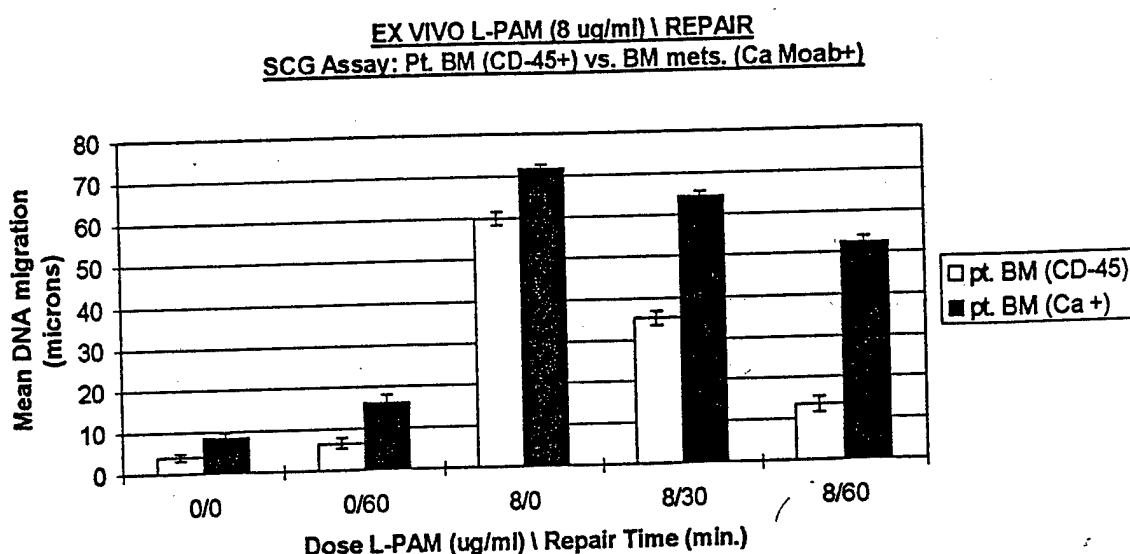
7.Artificial mixture experiments - - Ca Moab (+) cells in normal BM

Figure 9.

Artificial Mixture Experiments
Ca Moab (+) Cells / BM

8. Ex vivo melphalan exposures and repair - - SCG assay of CD-45 versus Ca Moab (+)

Figure 10.

EX VIVO L-PAM (8 ug/ml) \ REPAIR
SCG Assay: Pt. BM (CD-45+) vs. BM mets. (Ca Moab+)

Patient BM was immunotyped for normal cells (CD-45) and for cancer cells (Ca Moabs) by means of our phenotyping technique. The cells, in heterogenous mixtures, were either untreated or treated with melphalan at 8.0 ug/ml. They then were denied time to repair or allowed to repair for 30 or 60 minutes. The various combinations employed are presented on the X-axis of figure 10. Results are plotted as usual showing mean DNA migration in microns along with the SEMs. The results are encouraging. The labeling was clear, sufficient numbers of tumor cells were in evidence, and at the level of melphalan used, the cells of both types remained viable at levels permissive of accurate scoring for DNA damage.

The data in these studies raise a number of interesting points. The base level DNA damage is greater than what has been seen for normal BM. The apparent reason for this is that these patients had received induction therapies in the months prior to beginning their therapy in our program and persistent damage is evident. We has seen this in most of the one hundred plus patients tested using SCG. Tumor cells appear to have slightly more damage at baseline and also seem to be more sensitive to melphalan than the normal BM. The normal BM was more repair sufficient, repairing 79% of the initial damage compared with only 28% repaired among the metastatic cells. The viability of samples of cells in these same studies was ascertained and the scores provided in figures 11 & 12.
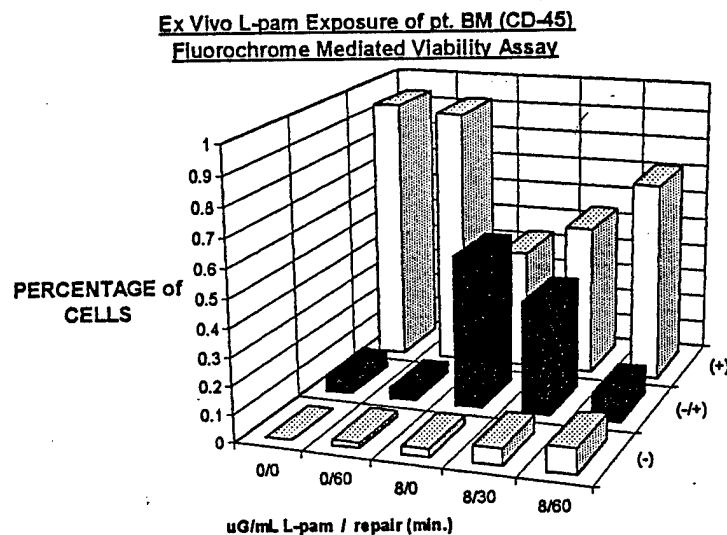
Figure 11 shows the viability scoring for the CD-45 cells and figure 12 shows the same for tumor from the same patient BM identified with Ca Moabs. The percentage of cells represented is plotted on the Y-axis and the X-axis shows the combinations of treatment and repair time. The levels of viability determined by the fluorochrome mediated viability assay (FMVA) are indicated to the right of the bars marked as (+) for live, (+/-) for dying and (-) for dead. In general, the viability data mirrors that of SCG for each cell set. Its

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importance lies in the fact that FMVA can be performed in minutes and the results used to make adjustment in the SCG assay being conducted.

Several observations are noteworthy. The cells that label as dead are not scoreable in the SCG assay and appear as artifacts known as 'clouds'. The dying cells are scoreable, often showing heavy damage that also seems to account for most of the fraction of cells that undergoes repair to the extent that repair occurs.
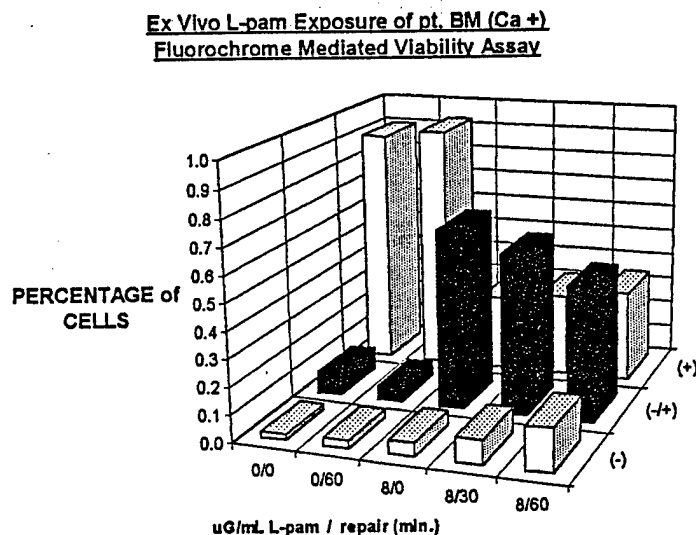
9. Ex vivo melphalan exposures and repair - - FMVA of CD-45 cells

Figure 11.

Ex Vivo L-pam Exposure of pt. BM (CD-45)
Fluorochrome Mediated Viability Assay

10. Ex vivo melphalan exposures and repair - - FMVA of Ca Moabs (+) cells

Figure 12.

Ex Vivo L-pam Exposure of pt. BM (Ca +)
Fluorochrome Mediated Viability Assay

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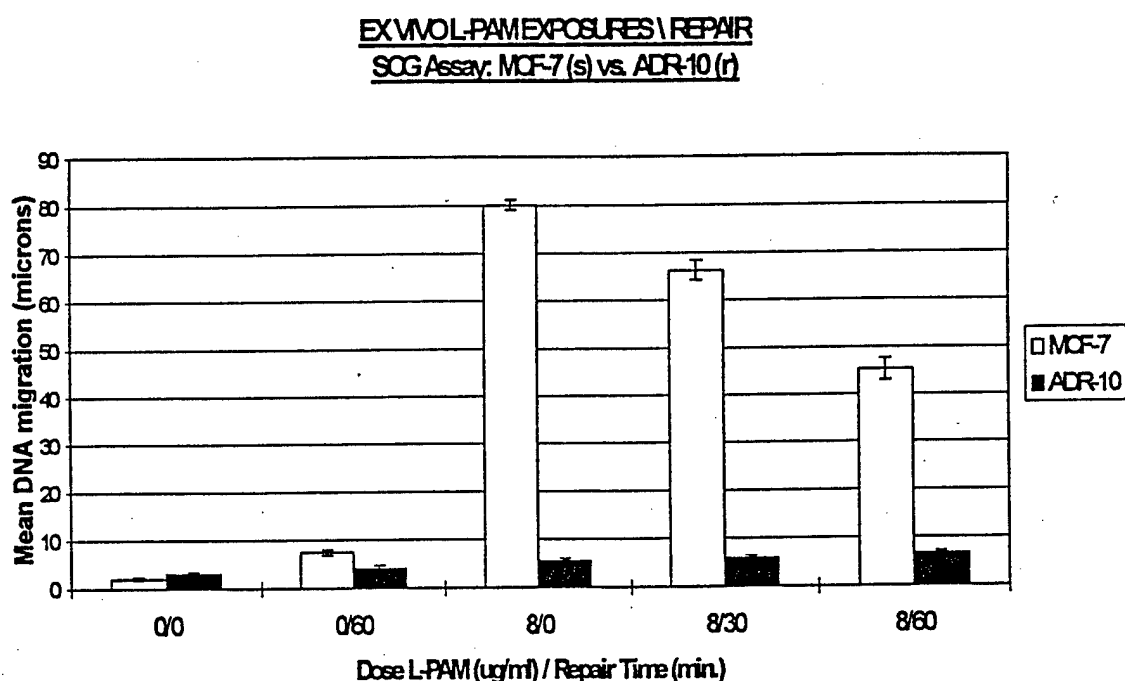## 11. Ex vivo melphalan exposures and repair - - SCG assay of MCF-7 versus ADR-10 cells

The following 2 figures refer to experiments conducted to test relative melphalan sensitivities of the cancer cell "cultured representative", MCF-7, the sensitive wild type and ADR-10, the MDR mutant line. In part, these cells are being tested to determine how well they actually mimic the behavior of the "true" wild type cell that we identify routinely in the BM as micrometastases. The major drawback to the use of these cells is that they require a high degree of care to grow. Because they are adherent cells, it was feared they might not respond to short term exposures in suspension as would the BM derived tumors. The inconvenience of removing the cells from the flasks, dissociating without damaging, etc. notwithstanding they have proven amenable to the testing requirements posed.

Figure 13.

EX VIVO L-PAM EXPOSURES \ REPAIR
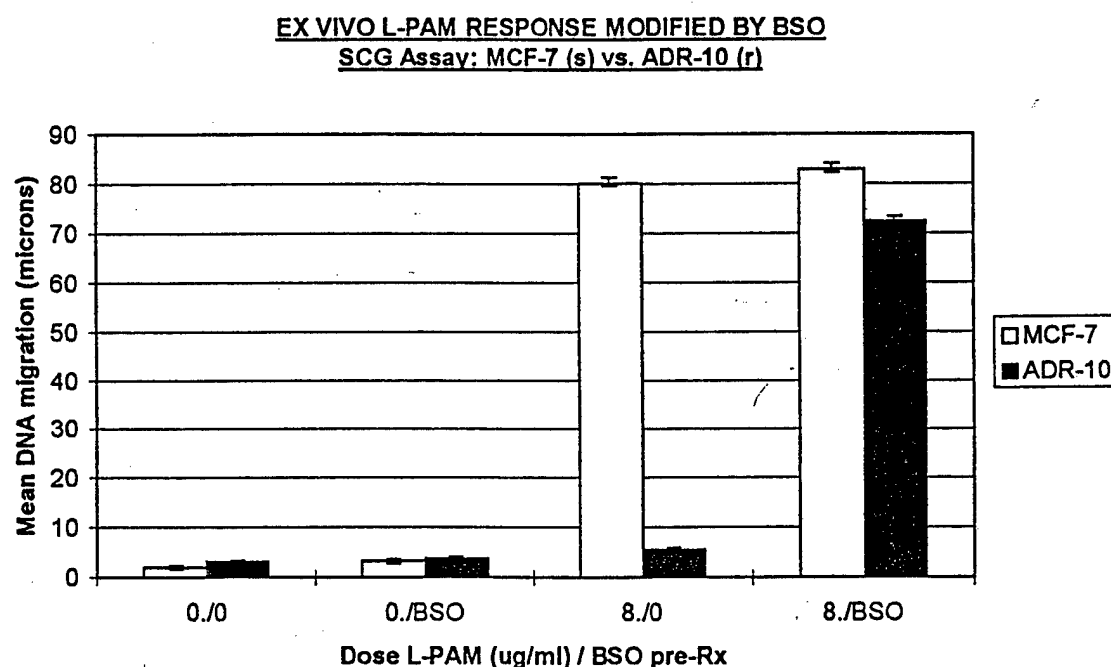**SCG Assay: MCF-7 (s) vs. ADR-10 (r)**

The testing scheme employed here is essentially the same as used in the previous 2 exposure and repair studies. Figure 13 shows results from SCG testing MCF-7 (white bars) and ADR-10 cells (black bars). The MCF-7 cells appear to be slightly more sensitive to initial DNA damage from melphalan exposure than were the micrometastatic tumor cells from patients but they also may be better at repairing the damage. The data above shows the MCF-7 line repaired 44% of initial damage compared with 28% observed for the tumor from patient BM. Clearly it will be most important to catalogue the range of such responses to determine individual variability in this regard. The ADR-10 cells were nearly completely insensitive to melphalan at the 8.0 ug/ml dose tested. This is apparently a new

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finding which is being followed up with more intensive studies into the biology of MDR by collaborators.

12. Ex vivo melphalan response modified by BSO - - SCG assay: MCF-7 vs. ADR-10 cells

Figure 14 shows results from experiments testing the ability of BSO to modify the melphalan sensitivity of MCF-7 and ADR-10 cells. As indicated on the X-axis, cells were either exposed with BSO (under conditions already described) or not and thereafter either exposed with 8.0 ug/ml melphalan or not. The results clearly indicate that BSO can render ADR-10 cells nearly as sensitive to melphalan as are the wild type, sensitive cells.
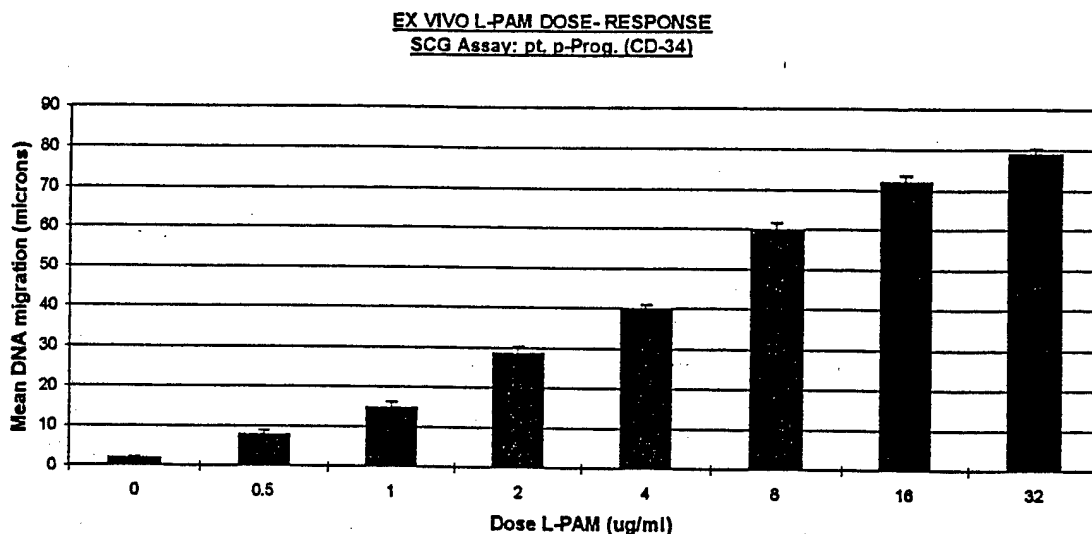
Figure 14.

EX VIVO L-PAM RESPONSE MODIFIED BY BSO
SCG Assay: MCF-7 (s) vs. ADR-10 (r)

p-Progenitor cells, acquired from patients by pheresis were tested for sensitivity to melphalan as assessed by SCG. As figure 15 shows, doses from 0.0 to 32.0 ug/ml melphalan were used to expose the p-prog. The results shows a smooth dose- response profile similar to those seen for other cells tested with this cell type being almost equal in sensitivity to the normal CD-5 cells tested. Again, one would point out that individual differences are to be expected and can only be ascertained with further testing and cataloguing of data.

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13. Ex vivo melphalan dose- response – SCG assay of normal CD-45 cells

Figure 15.

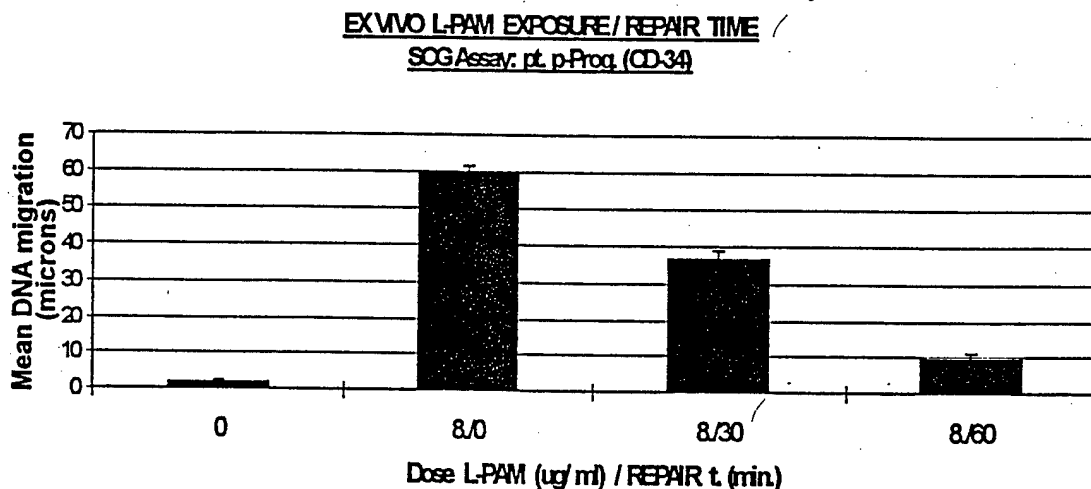**EX VIVO L-PAM DOSE- RESPONSE**
SCG Assay: pt. p-Prog. (CD-34)

14. Ex vivo melphalan exposures and repair - - SCG assay of p-progenitors

Figure 16 presents results of testing patient p-prog for DNA damaging effects at a melphalan dose level of 8.0 ug/ml and ability to repair the damage.

Figure 16.

EX VIVO L-PAM EXPOSURE / REPAIR TIME
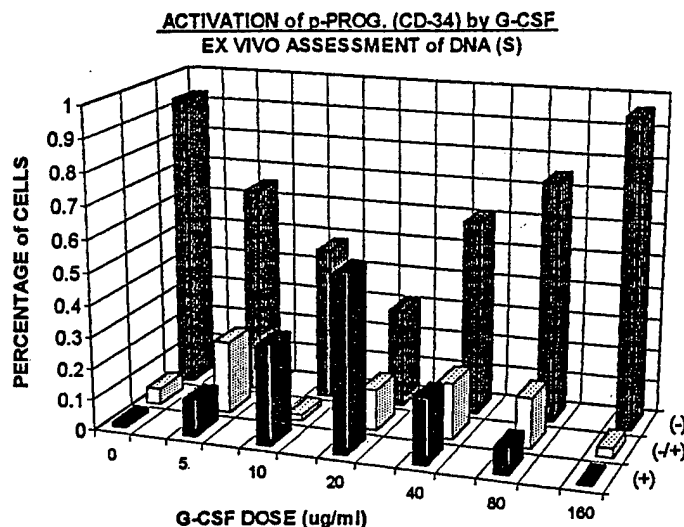**SCG Assay: pt. p-Prog. (CD-34)**

The untreated p-prog showed a low level of base line damage (although they come from patients whom were treated previously with induction therapy). They repair well. After 30 minutes they repaired an average of 37% of initial damage and a further 46% by 60 minutes. The total repair after an hour averaged 83% compared with 87% for CD-5 cells aliquoted from a normal individual, cryopreserved and tested repeatedly over a period of months.

The next set of experiments in this series was designed to measure ex vivo responses of p-prog to G-CSF. A dose- response curve was generated as shown in figure 17. This was done using doubling doses, in ug/ml, from 5.0 to 160.0 and allowing incorporation of added BrdU, plating to slides and processed for SCG as usual. The slides were then stained with FITC-X-BrdU and scored.

Figure 17.

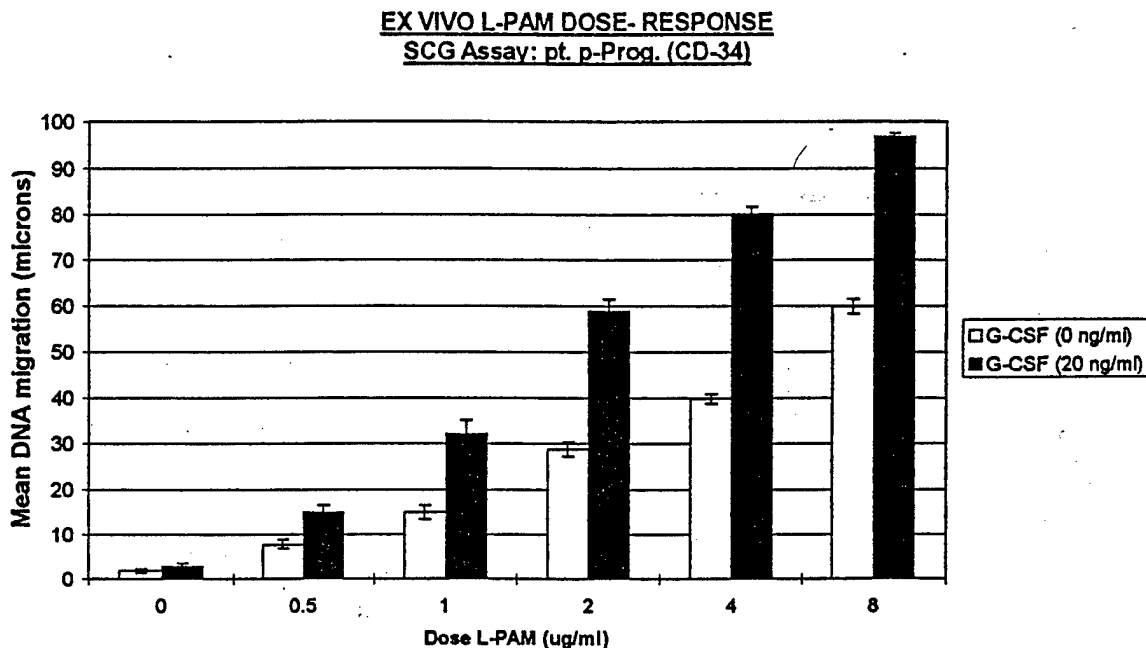ACTIVATION of p-PROG. (CD-34) by G-CSF
EX VIVO ASSESSMENT of DNA (S)

Using the BrdU method of labeling for DNA synthetic cells positive nuclei fluoresce green on a background of the red ethidium bromide used to label DNA in SCG testing. The nuclei were scored using a -/ +/- /+ system. This method performs well especially when the bias is placed against the positive and negative cells by scoring +/- unless there is no doubt whatsoever that a cell is -/ or +/ . When scoring SCG and proliferative index together it is actually easier score accurately because, even in the absence of a discernible tail, the DNA has unwound and the nuclear diameter expanded roughly 2X. The BrdU labeling is easier to visualize in this state. Cells that are damaged and proliferative appear to be yellow-orange as they fluoresce both red and green. Not surprisingly given this information, the greater the migration length ,the easier it is to assign a proliferation state score. Again, one can afford to score conservatively with utmost certainty because finding 100 scorable cells on a slide containing 10^4 or more is easily accomplished.

The data in figure 17 show results from testing the p-prog of one patient. Further studies (not shown here) have provided similar results. The optimal dose determined for this patient is 20.0 ug/ml. Bell-shaped curves appear around this point, including one that is inverted mirroring the positive one in the case of the unstimulated portion. It is likely that this dose will prove optimal for stimulating p-prog from all patients. This will depend upon the extent of variability of response discovered on testing more patients. In our experience with immunocompetency testing we have found, in, that doses well outside the "mean effective dose" are sometimes required to produce an expected response. If

necessary we will run new titration curves on p-prog from individuals. The data show that, at doses above 20.0 ug/ml, the effect is inhibitory and possibly toxic. Further testing using FMVA and SCG should sort this out.

16. Ex Vivo melphalan dose- response - - SCG assay of p-prog: G-CSF @ 0.0 ug/ml versus 20.0 ug/ml

Figure 18.

EX VIVO L-PAM DOSE- RESPONSE
SCG Assay: pt. p-Prog. (CD-34)

An SCG assay was run on the same set of patients' p-prog specimens tested and results reported in figure 18. In this case p-prog were either treated with 0.0 (white bars) or 20.0 (black bars) ug/ml G-CSF. They then were titrated against melphalan doses of from 0.0 to 8.0 ug/ml as shown on the X-axis and SCG assayed. At every dose of melphalan, cells stimulated to DNA (S) with G-CSF expressed enhanced degrees of DNA damage compared with the set not treated with G-CSF. This may be reminiscent of the commonly held belief that proliferative cells, e.g. tumor cells in vivo, are more sensitive to cytotoxic therapy than are their quiescent counterparts. Experiments to assess the effects of G-CSF activation of p-prog on their DNA repair ability are now scheduled. Also scheduled are tests to determine ,in the ex vivo setting, whether G-CSF similarly affects BM tumor cells. This may become important for theoretical purposes if tumor growth is enhanced in some respect.

Data have been collected, to date, on 34 patients on the first day of their first High Dose treatment cycle. At the time of testing the patients had received only melphalan infused over a period of 7 h. These patients continued their treatment on the ICAM, not the Melphalan Only protocol. The disposition of melphalan has been evaluated by HPLC as described. In general, melphalan disappears rapidly from plasma displaying a median overall elimination half-life of 18 minutes and reaching undetectable concentrations within 6 hours. A two compartment open model with zero order input and first order elimination adequately described the pattern of disposition in all patients evaluated. Average systemic
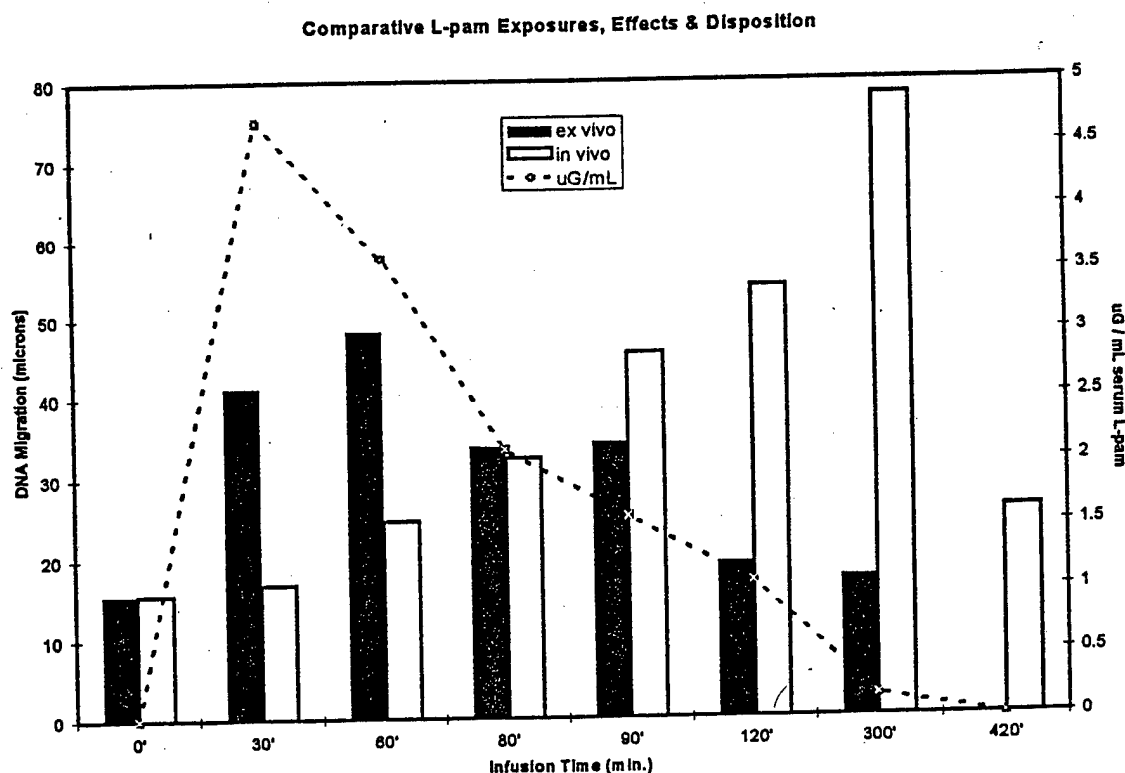
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exposure (Cmax and AUC) increased with dose; however, a 2.2 fold range in drug clearance resulted in overlapping systemic exposures of individual patients across dosage levels.

At the times of sampling, treatment under the protocols, Melphalan Only and ICAM, is identical. The comparative tests performed were: 1.) SCG on patient CD-5 cells (CD-15 cells were also tests but results are not shown here) (white bars); 2.) SCG on the normal control CD-5 cells exposed with plasma drawn from the patient at times indicated (black bars) and 3.) pharmacokinetic measurements by HPLC assay also at the times indicated on the X-axis (dotted line).

C. Routine testing

1. SCG assays on 1.) in vivo melphalan exposures in pt. CD-5 cells, 2.) ex vivo plasma (from same pt) exposures on normal CD-5 cells & 3.) HPLC counts on same pt. Plasma samples

Figure 19.

Comparative L-pam Exposures, Effects & Disposition

The data shown in figure 19 are based on tests from one patient in a continuing series.

Owing to data sharing restrictions defined by grant contracts held by collaborators in the BMTP, data resulting from pharmacokinetic studies can not be released, at present, beyond the "representative" data shown here. The entirety of the data is currently being evaluated after the recent closure of the ICAM Study (described more fully in the proposal appendix). It has been replaced with another from which we are also drawing patients for

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our studies. Our SCG and viability data are to be evaluated along with myriad endpoint information to be processed for correlations.

The data shown here are similar to those collected for other patients in the series in that there is usually a lag in the appearance of DNA damage above baseline in the CD-5 cells exposed in vivo (white bars). Damaged cells generally express themselves in SCG after at or after 60 minutes and after peak levels according to the machine counts have already been passed and are dropping. This is an instance of a major gap in our knowledge with reference to latency and patency periods of damage expression in vivo An understanding of such activities might be helpful in developing therapeutic approaches, possibly through real-time monitoring of individuals undergoing treatment.

The ex vivo plasma exposure studies have also yielded interesting results. The indicator cells show damage that seems to follow the curve established by machine measurements. This finding is expected in theory, but nevertheless is gratifying to observe because one originally suspected decomposition of the drug might render it biologically undetectable. Fortunately, so far at least, the test cells as tested in the SCG assay are sensitive enough to provide useful results from the specimens which contain sufficient active drug. We are anxious to determine, for each patient individually, to what degree the HPLC results predict the biomarker finding. There are always questions looming concerning the relationships between measures of drug present in various forms and levels of biological activity.

IV. Conclusions

A. Project status

The project has been completed, and the results are interesting and vague further questions. Clearly the patients bone marrow progenitor cells show more DNA damage than normal controls. In addition, the single cell gel assess was able to detect the increased DNA damage of tumor cells contaminating the bone marrow. As a result of this project, the Duke University Bone Marrow Transplant program is including a drug resistance section in their resubmission for their Program Project Grant. We will investigate gluethione levels, gluethione S-thrase, isoenzymes, as well as different mechanisms of DNA repair, and modifiers of drug resistance of DNA repair in an attempt to increase the tumor cell killing and decrease the toxicity to normal hemotopoietic progenitor cells.

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Our results to date are encouraging in that, with the above exception noted, the battery of test methods is in place and operating well. All tasks proposed in the S.O.W. and listed below are now accomplished or underway according to plan.

1.Tasks list

Task 1, titrate for relevant dose, check detection limits,
    a. G-CSF: expose p-progenitors & use proliferation assay
    b. plasma: expose normal BM & CAMA-1 (artificial mixes) & SCG\ repair
    c. melphalan: expose normal BM & CAMA-1 (artificial mixes) & SCG\ repair
Task 2, titrate for relevant dose, test for GSH (in BM)
       test effect of GSH depletion & if BSO protects naked DNA
    a. BSO doses: to normal BM & CAMA-1 (mix),melphalan expose & SCG\ repair
    b. BSO doses: to normal BM & CAMA-1 (mix),melphalan expose & SCG\ naked
Task 3, apply in vivo exposures test battery to each patient accrued,
Task 4, apply in vitro exposures test battery to each patient accrued,

2. Deliverables list

It is likely that our hypotheses can be successfully tested. One believes that, though deliverables are off schedule, they now can be accomplished within a reasonable time frame. The original list of proposed deliverables follows along with notes on progress to date and changes that seem reasonable at this time (titles are working versions):

D1, = Article: SCG Assessment of in vitro DNA damage and repair in normal & tumor cells after melphalan exposure. Manuscript in progress.
D1a, Article: SCG Assessment of Response Modification in MDR Cell Lines Exposed with Arsenic, 4-HC or Melphalan. Manuscript submitted for clearance.
D2, = Article: SCG Assessment of in vitro DNA damage and repair in normal & tumor cells after melphalan exposure II. Role of proliferative state in determining relative sensitivity. Data being prepared for publication. May combine D2 and D3
D3, = Article: SCG Assessment of in vitro DNA damage and repair in normal & tumor cells after melphalan exposure III. Role of GSH in cellular sensitivity.
D4 , = Project Progress Report & Article: Preliminary results from a longitudinal study of individual breast cancer patients receiving high-dose melphalan and progenitor cell support: Serial assessments of therapeutic vs. toxic effects using a genotoxicity / cytotoxicity battery. May combine D4 & D5
D5, = Final Project Report & Article: A longitudinal study of individual breast cancer patients receiving high-dose melphalan and progenitor cell support: Serial assessments of therapeutic vs. toxic effects using a genotoxicity / cytotoxicity battery.

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